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(54) **HUMAN TPA PRODUCTION USING VECTORS CODING FOR DHFR PROTEIN**

1988, now abandoned, which is a continuation of application No. 06/459,153, filed on Jan. 19, 1983, now abandoned.

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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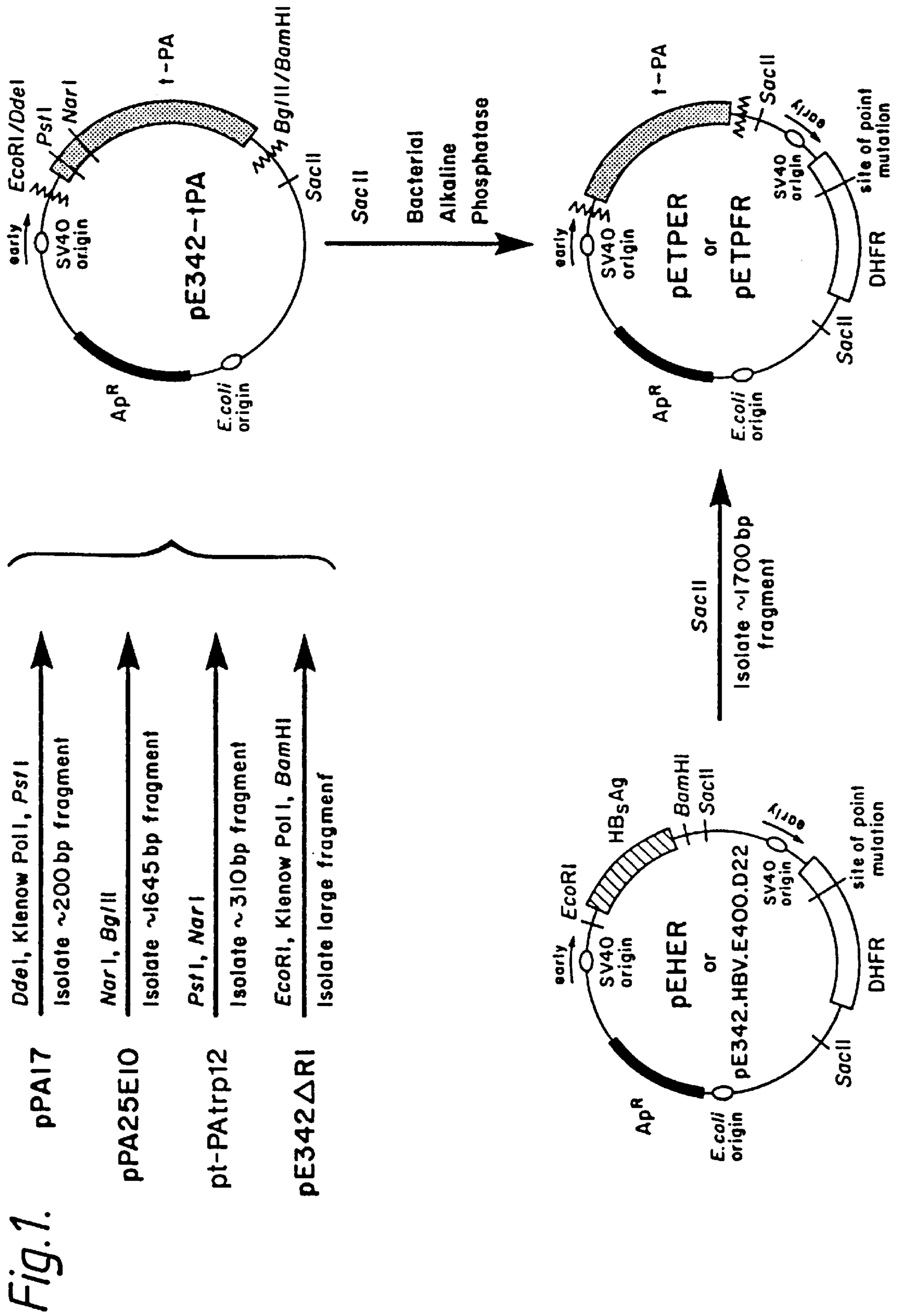
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(57) **ABSTRACT**

(63) Continuation of application No. 08/450,874, filed on May 26, 1995, now Pat. No. 5,849,574, which is a continuation of application No. 08/162,354, filed on Dec. 3, 1993, now Pat. No. 5,424,198, which is a division of application No. 07/663,103, filed on Feb. 28, 1991, now Pat. No. 5,268,291, which is a continuation of application No. 07/499,209, filed on Mar. 22, 1990, now Pat. No. 5,010,002, which is a continuation of application No. 07/149,990, filed on Jan. 27,

A method for producing tissue plasminogen activator (t-PA) in eukaryotic host cells is disclosed. Enhanced levels of t-PA production are obtained by co-amplification of the t-PA gene through treatment of cultures transformed with mutant or wild type DHFR with methotrexate.

5 Claims, 1 Drawing Sheet



HUMAN TPA PRODUCTION USING VECTORS CODING FOR DHFR PROTEIN

This is a continuation of application Ser. No. 08/450,874, filed May 26, 1995 now U.S. Pat. No. 5,849,574, which is a continuation of application Ser. No. 08/162,354, filed Dec. 3, 1993 now U.S. Pat. No. 5,424,198, which is a division of application Ser. No. 07/663,103, filed Feb. 28, 1991, now U.S. Pat. No. 5,268,291, which is a continuation of application Ser. No. 07/499,209, filed Mar. 22, 1990, now U.S. Pat. No. 5,010,002, which is a continuation of application Ser. No. 07/149,990, filed Jan. 27, 1988, abandoned, which is a continuation of application Ser. No. 06/459,153, filed Jan. 19, 1983, abandoned. Reference is hereby made under 35 USC 120/121 to U.S. Ser. No. 06/374,860, filed May 5, 1982, U.S. Ser. No. 06/398,003, filed Jul. 14, 1982, and U.S. Ser. No. 06/483,052, filed Apr. 7, 1983.

BACKGROUND OF THE INVENTION

The invention herein relates to the production of human tissue plasminogen activator (tPA) in a transformant host cell culture. More specifically, the invention relates to vectors, cells, and methods of producing tPA in conjunction with expression of the sequences for coding for dihydrofolate reductase (DHFR) protein in such cells.

The production of tPA using recombinant techniques has been disclosed in U.S. Application Ser. No. 397,987, filed Jul. 14, 1982 which is a continuation in part of U.S. Ser. No. 374,860 filed May 5, 1982; the contents of both applications are incorporated herein by reference. These applications describe the construction of plasmids containing the coding sequences for tPA, and describe the activity and utility of tPA so produced.

It is also been found, as set forth in co-pending applications Genentech Docket No. 100/92, and 100/140, filed on even date herewith, and incorporated herein by reference, that a DNA sequence encoding for a DHFR protein can be utilized as a marker for transfection of a sequence coding for a desired heterologous protein in suitable host cells. The DHFR sequence can also be used as a secondary sequence permitting control of the production of the desired protein. These applications disclose such a use, both of wild type DHPA, and of a mutant DHFR which is resistant to methotrexate.

A problem frequently encountered in the production of polypeptides in a foreign host is the necessity to have some mechanism to regulate, usually to enhance, the production of the desired protein. In the case of tPA, which forms the subject matter of this invention, a secondary coding sequence comprising DHFR which is affected by an externally controlled parameter, such as methotrexate, is utilized to permit control of expression by control of the methotrexate (MTX) concentration.

Methotrexate is a drug which is normally fatal to cells capable of its uptake. However, certain cells are able to grow in the presence of controlled levels of MTX. One of the several mechanisms whereby methotrexate resistance is effected is that whereby amplification of the gene coding for the DHFR coding sequence is stimulated (Schimke, Robert T. et al, *Science*, 202:1051 (1978); Biedler, J. L. et al, *Cancer Res.* 32:153 (1972); Chang, S. E., et al, *Cell*, 7:391 (1976)).

It has further been shown that amplification of the gene for DHFR may further cause amplification of associated sequences which code for other proteins. This appears to be the case when the associated protein is hepatitis B surface antigen (HBsAg) (Christman, J. et al, *Proc. Natl. Acad. Sci.*,

79:1815 (1982)); the *E. coli* protein XGPRT (Ringold, Gordon, et al, *J. Molec. and Appl. Gen.*, 1:165 (1981)); and an endogenous sequence from a DHFR/SV40 plasmid combination (Kaufman, R. F. et al, *J. Molec. Biol.*, 159:601 (1982)).

Other mechanisms for conferring methotrexate resistance include diminution of the binding affinity of the DHFR protein, so that it is less susceptible to methotrexate (Flintoff, W. F. et al, *Somat. Cell Genet.*, 2:245 (1976)) but in this instance, amplification appears to occur as well.

Thus it would appear that the genes both for wild type DHFR and for DHFR which is resistant to MTX by virtue of its own decreased binding capacity are amplified by the presence of MTX. Hence, in principle, the invention herein concerns using the impact of DHFR sequence amplification on associated protein coding sequences to provide a control mechanism which permits enhanced expression levels of tPA sequences in the presence of MTX, or by virtue of prior treatment of transformed cells with MTX.

As described in U.S. Ser. No. 397,987, tPA is a fibrinolytic substance which can be recovered from human melanoma cells (EPO Patent Application Publ. No. 0041766). This product has been isolated and characterized [Weiman et al, *The Lancet*, II (8250):1018 (1981)]. Its fibrinolytic activity is analogous to that of two commercially available proteins, streptokinase and urokinase, which are indicated for the treatment of acute cardiovascular diseases such as myocardial infarct, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion and other venous thrombosis. The etiological basis for these diseases is apparently either a partial or total occlusion of a blood vessel by a blood clot. Thus traditional anticoagulant therapy for example, treatment with heparin or coumarin, is not effective as it will merely prevent the formation of further clots, but not result in the dissolution of clots already formed. The fibrinolytic agents, streptokinase, urokinase, and plasminogen activator all operate similarly. They convert the inactive precursor plasminogen into plasmin which is capable of dissolving the fibrin of which these clots are composed. Plasminogen activator has a high affinity for fibrin, and thus preferentially activates plasminogen associated with the fibrin desired to be dissolved. On the other hand, streptokinase and urokinase do not; hence, much of the plasmin formed is formed in circulating blood and is neutralized before it can reach the targeted clot. Furthermore, as these compounds create circulating rather than fibrin bound plasmin, other clotting factor proteins in circulation such as fibrinogen, Factor V, and Factor VIII are also attacked by the activated protein causing a hemorrhagic potential. Furthermore, streptokinase is strongly immunogenic.

Plasminogen activator overcomes the foregoing difficulties by specifically attacking plasminogen already bound to fibrin. The present Invention concerns a method of increasing and controlling the production of this valuable protein in recombinant cultures by effecting control on amplification of the sequence for DHFR protein.

SUMMARY OF THE INVENTION

In one aspect, the invention herein concerns plasmids which contain coding sequences for human tissue plasminogen activator (tPA) and a DHFR protein, and which are effective in expressing both of them. In another aspect, the invention concerns cells transformed with these vectors.

In other aspects, the invention also concerns methods for producing tPA by taking advantage of the environmentally controlled response of DHFR coding sequences co-transfected with the tPA sequence, and the tPA so produced.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a schematic of the construction of the exemplified DHFR (mutant or wild type)/tPA encoding plasmids.

DETAILED DESCRIPTION

A. Definitions

As used herein:

Human "tissue plasminogen activator" (tPA) is a fibrinolytic protein as described in U.S. Ser. No. 272,093, filed Jun. 11, 1980 which is a continuation in part of Ser. No. 183,638 filed Sep. 3, 1980, both incorporated herein by reference.

"DHFR protein" refers to a protein which is capable of the activity associated with dihydrofolate reductase (DHFR) and which, therefore, is required to be produced by cells which are capable of survival on medium deficient in hypoxanthine, glycine, and thymidine (-HGT medium). In general, cells lacking DHFR protein are incapable of growing on this medium, cells which contain DHFR protein are successful in doing so.

"Cells sensitive to MTX" refers to cells which are incapable of growing on media which contain the DHFR inhibitor methotrexate (MTX). Thus, "cells sensitive to MTX" are cells which, unless genetically altered or otherwise supplemented, will fail to grow under ambient and medium conditions suitable for the cell type when the MTX concentration is 0.2 $\mu\text{g}/\text{ml}$ or more. Some cells, such as bacteria, fail to exhibit MTX sensitivity due to their failure to permit MTX inside their cell boundaries, even though they contain DHFR which would otherwise be sensitive to this drug. In general, cells which contain, as their DHFR protein, wild type DHFR will be sensitive to methotrexate if they are permeable or capable of uptake with respect to MTX.

"Wild type DHFR" refers to dihydrofolate reductase as is ordinarily found in the particular organism in question. Wild type DHFR is generally sensitive in vitro to low concentrations of methotrexate.

"DHFR protein with low binding affinity for MTX" has a functional definition. This is a DHFR protein which, when generated within cells, will permit the growth of MTX sensitive cells in a medium containing 0.2 $\mu\text{g}/\text{ml}$ or more of MTX. It is recognized that such a functional definition depends on the facility with which the organism produces the "DHFR protein with low binding affinity for MTX" as well as upon the protein itself. However, as used in the context of this invention, such a balance between these two mechanisms should not be troublesome. The invention operates with respect to conferring the capability of surviving these levels of MTX, and it is not consequential whether the ability to do so is impacted by increased expression in addition to the innate nature of the DHFR produced.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified DNA code disposed therein is included in this term as it is applied to the specified sequence. In general, expression vectors of utility in recombinant DNA techniques

are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, tPA produced in the amounts achieved by virtue of this transformation, rather than in such lesser amounts, or, more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

B. Detailed Description

B.1 Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F λ , prototrophic, ATCC No. 27325), bacilli such as *Bacillus subtilis*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR 322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2:95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β -lactamase (penicillinase) and lactose promoter systems (Chang et al, *Nature*, 275:615 (1978); Itakura, et al, *Science*, 198:1056 (1977); (Goeddel, et al *Nature* 281:544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic Acids Res.*, 8:4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, *Cell* 20:269 (1980)).

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in

Saccharomyces, the plasmid YRp7, for example, (Stinchcomb, et al, *Nature*, 282:39 (1979); Kingsman et al, *Gene*, 7:141 (1979); Tschemper, et al, *Gene*, 10:157 (1980)) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.*, 255:2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.*, 7:149 (1968); Holland, et al, *Biochemistry*, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (Holland, *ibid.*). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MOCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences. It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those sequences exemplified.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature*, 273:113 (1978) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the

desired gene sequence, provide such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

B.2 Selection of Cell Lines

In selecting a preferred host cell for transfection by the vectors of the invention, it is appropriate to select the host according to the type of DHFR protein employed. If wild type DHFR protein is employed, it is preferable to select a host cell which is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selective medium which lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. (USA)* 77:4216 (1980), incorporated-herein by reference.

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR resistant cells. Because the mutant DHFR is resistant to methotrexate, MTX containing media can be used as a means of selection provided that the host cells are themselves methotrexate sensitive. Most eukaryotic cells which are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 ATCC No. CCL 61.

The example which is set forth hereinbelow describes use of CHO cells as host cells, and expression vectors which include the SV40 origin of replication as a promoter. However, it would be well within the skill of the art to use analogous techniques to construct expression vectors for expression of desired protein sequences in alternative eukaryotic host cell cultures.

B.3 Methods Employed

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52:546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci. (USA)*, 69:2110 (1972).

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and relegated in the form desired to form the plasmids required.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 μ g plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μ l of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, S., et al, *Nucleic Acids Res.*, 8:4057 (1980) incorporated herein by reference.

For ligation approximately equimolar amount of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.6 µg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

The ligation mixture was used to transform *E. coli* K12 strain 294 (ATLC 31446), and successful transformants were selected by ampicillin resistance. Plasmids from the transformants were prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.*, 9:309 (1981) or by the method of Maxam, et al, *Methods in Enzymology*, 65:499 (1980).

Amplification of DHFR protein coding sequences is effected by growing host cell cultures in the presence of approximately 20–500,000 nM concentrations of methotrexate, a competitive inhibitor of DHFR activity. The effective range of concentration is highly dependent, of course, upon the nature of the DHFR gene, protein and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds which inhibit DHFR could also be used. MTX itself is, however, convenient, readily available and effective.

B.4 Results Obtainable

The methods of the invention permit the production in host cell cultures of antigenically active tPA protein in amounts greater than 0.1 pg per cell per day. With suitable application of amplifying conditions, amounts greater than 20 pg can be obtained. Stated in alternate terms, gene expression levels resulting in production of more than 9×10^{-6} units, or, with suitable amplification, more than 18×10^{-5} units of tPA activity are achieved.

C. Examples

The following examples are intended to illustrate but not to limit the invention. In the examples here, a CHO cell line suitable for the type of DHFR protein coding sequence to be introduced was employed as a host cell culture in each case. However, other eukaryotic and prokaryotic cells are suitable for the method of the invention as well.

C.1 Production of tPA Using DHFR Protein with a Low Binding Affinity for MTX

C.1. A Vector Construction

The sequence encoding human tissue plasminogen activator (tPA) is inserted, an expression plasmid for a mutant DHFR with low binding affinity for MTX, described in copending application Genentech Docket No. 100/140, incorporated herein by reference, by the following procedure (see FIG. 1):

cDNA plasmids encoding tPA have been described by Goeddel et al, application Ser. No. 374,860, filed May 5, 1982, which is hereby incorporated by reference. Three fragments from overlapping tPA plasmids, pPA25E10, and pPA17, are pΔRIPA^o were prepared as follows: Plasmid pPA17 was digested with Dde I, filled in using Klenow DNA polymerase 1, and subcut with Pst 1; the approximately 200

bp fragment containing 5' terminal tPA sequence thus generated was isolated. The second tPA fragment was obtained by digesting pΔRIPA^o with Pst I and Nar I and isolating the approximately 310 bp fragment. The third tPA fragment was obtained by digesting pPA25E10 with Nar I and Bgl II and isolating the approximately 1645 bp fragment which contains, in addition to much of the tPA coding region, some 3' non-translated sequences.

Plasmid p342E which expresses HBV surface antigen (also referred to as pHBs348-E) has been described by Levinson et al, patent application Ser. No. 326,980, filed Dec. 3, 1981, which is incorporated herein by reference. pE342 is modified by digesting with trace amounts of Eco RI, filling in the cleaved site using Klenow DNA polymerase I, and ligating the plasmid back together, thus removing the Eco RI site preceding the SV40 origin in pE342. The resulting plasmid, designated pE342ΔR1, is digested with Eco RI, filled in using Klenow DNA polymerase I, and subcut with Bam HI. After electrophoresing on acrylamide gel, the approximately 3500 bp fragment is electroeluted, phenol-chloroformed, and ethanolated as above.

The thus prepared p342E 3500 bp vector, and above described tPA fragments comprising approximately 2160 bp were ligated together using standard techniques. A plasmid containing the three tPA encoding fragments in the proper orientation was isolated, characterized, and designated pE342-tPA. This plasmid was digested with Sac II and treated with bacterial alkaline phosphatase (BRL). To provide the DHFR sequence (along with control sequences for its expression) an approximately 1700 bp fragment was generated by SacII digestion of pEHER. (pEHER is a plasmid expressing mutant DHFR described in copending Genentech docket no. 100/140.) This fragment was ligated into the pE342-tPA plasmid to create pETPAER400, a plasmid which is analogous to pEHER except that the HBsAg coding region has been replaced by the cDNA sequences from tPA.

C.1.B Expression and Amplification of the tPA Sequence

pETPAER400 (pETPER) was transfected into both dhfr (CHO-DUX B11) obtained by permission from Urlaub and Chasin, and DHFR+ CHO-K1 (ATCC CCL61) cells by the method of Graham and Van der Eb (supra). Transformed dhfr cells were selected by growth in glycine, hypoxanthine and thymidine deficient medium. Transformed DHFR+ cells were selected by growth in ≥ 100 nM MTX. Colonies which arose on the appropriate selection medium were isolated using cloning rings and propagated in the same medium to several generations.

For amplification cells from the colonies are split into media containing 5×10^4 , 10^5 , 2.5×10^5 , 5×10^5 , and 10^6 nM MTX and passaged several times. Cells are plated at very low (10^2 – 10^3 cells/plate) cell densities in 10 cm dishes and the resulting colonies are isolated as usual.

C.1.C Assay Methods

Expression of tPA in the transfected amplified colonies may conveniently be assayed by the methods set forth in U.S. application Ser. No. 397,987. Briefly, for quantitative assay, the medium or extract to be tested is placed in a solution containing plasminogen, and the amount of plasmin formed is measured by monitoring the cleavage of a chromogenic substrate such as S2251, Kabi Group Inc., Greenwich, Conn. An aliquot of the sample is mixed with 0.1 ml of 0.7 mg/ml plasminogen (in 0.5M Tris-HCl, pH 7.4, containing 0.012M NaCl) and the volume adjusted to 0.15 ml. The mixture is incubated at 37° C. for ten minutes, 0.35 ml of S2251 (1.0 nM solution in the above buffer) is added and the

reaction continued for 30 minutes at 37° C. Acetic acid (25 μ l) is added to terminate the reaction. The samples are centrifuged and the absorbance at 405 nm is measured. Quantitation of the amount of activity is obtained by comparison with a standard urokinase solution. The assay conditions for detection of a full length plasminogen activator were modified by the addition of fibrinogen (0.2 mg) to the solution. Fibrinogen results in a stimulation of the activity of plasminogen activator observed, therefore resulting in somewhat elevated levels of activity. Activity was recorded in Plough units, wherein 90,000 Plough units is equal to the activity exhibited by 1 mg of purified tissue plasminogen activator.

Coamplification of DHFR and tPA sequences is assayed by isolating DNA from confluent monolayers of amplified colonies as follows: Confluent monolayers in 150 mm plates are washed with 50 ml sterile PBS and lysed by the addition of 5 ml of 0.1 percent SDS, 0.4M CaCl₂, 0.1M EDTA, pH 8. After 5–10 minutes, the mixture is removed, phenol extracted, chloroform extracted, and ethanol precipitated. The DNA is resuspended in 1 ml (per 150 mm plate) 10 mM Tris pH 8, 1 mM EDTA (TE), RNase added to 0.1 mg/ml, and the solution incubated 30 minutes at 37°. SDS is then added to 0.1 percent and pronase (Sigma) is added to 0.5 mg/ml. After 3–16 hours incubation at 37°, the solution is again phenol extracted, chloroform extracted, and ethanol precipitated as usual. The DNA pellet is resuspended in 0.5 ml water and digested with restriction enzymes as per the standard protocol. Approximately 5–10 μ g of digested DNA is electrophoresed in an agarose gel [1 percent agarose in Tris—acetate buffer (40 mM Tris, 1 mM EDTA, made to pH 8.2 with acetic acid)]; Crouse, et al, *J. Biol. Chem.*, 257:7887 (1982)). After bromphenol blue dye had migrated $\frac{2}{3}$ of the way down the gel, the gel is removed and stained with ethidium bromide. After visualizing the DNA with ultraviolet light, the gel is treated with HCl, NaOH, and NaCl-Tris and transferred to nitrocellulose filters according to the procedure of Southern (*J. Mol. Biol.* 98:503.(1975)). The filters are then hybridized with a nick translated probe made from the 1700 bp SacII fragment of pEHER (prepared and hybridized as described above), -or from the approximately 1970 bp Bgl II fragment of pETPER.

C.2 Production of tPA in Conjunction with Wild Type DHFR Protein

C.2.A. Vector Construction

In a manner exactly analogous to that used in the construction of pETPER, a plasmid containing the DNA sequence encoding wild type DHFR, pETPFR, was constructed. The construction was exactly as described in Example C.1.A except that in place of plasmid pEHER as a source for the DHFR protein gene sequence, the plasmid pE342.HBV.E400.D22 described in copending Genentech Docket No. 100/92 was substituted. The plasmid pE342.HBV.E400.D22 is exactly the same as pEHER except for a single base pair difference between wild type and mutant DHFR. Thus the resulting plasmid pETPFR is analogous in every way to pETPER except that the DNA sequence encoding for wild type DHFR is substituted for that of the mutant.

C.2.B Expression of tPA sequence

pETPFR was used to transfect DHFR deficient CHO cells (Urlaub and Chasin (supra)) using the calcium phosphate precipitation method of Graham and Van der Eb. Twenty-one colonies which arose on the selective medium (-HGT) were assayed by detection of plasmin

formation as assessed by the digestion of fibrin in an agar plate containing fibrin and plasminogen, described by Granelli-Piperno, et al, *J. Exp. Med.*, 148:223 (1978).

Four of the best positive clones were then assayed quantitatively for plasmin formation on a per cell basis according to the method set forth in C.1.C.

Upon such quantitative determination it was found that the four clones tested exhibited the same or comparable tPA secretion into the medium, determined as units/cell/day. Subclones were prepared by transferring inocula from two of the clones into separate plates containing -HGT medium. Two of the resulting subclones, 18B and 1 were used for further analysis.

C.2.C Amplification and tPA Production Levels

The above subclones were plated at 2×10^5 cells per 100 mm plates in 50 nM MTX to promote amplification. Those cells which survived, when assayed as described above, gave, in all cases, about 10 times the unamplified amount of plasminogen activator activity. Two of these clones were chosen for further study and were named 1–15 and 18B–9.

Subclone 1–15 was further amplified by seeding 2×10^5 cells in 100 mm plates containing 500 nM MTX. Assay of the cells thus amplified yielded a further increase (of about 3 fold) in tPA production; when assayed quantitatively by the method of C.1.C, levels were in the range of 7×10^{-4} units/cell/day. A portion of these amplified cells was then transferred and maintained in the presence of 10,000 nM MTX. Subclones of 1–15, and 18B–9 were further tested after being maintained for approximately 1–2 months at the conditions specified in Table 1.

TABLE 1

Cell Line	Growth Conditions	ng tPA/cell/day*
1-15 ₅₀₀	500 nM MTX	28.5×10^{-3}
1-15 ₅₀₀	(-HGT medium, no MTX)	8.3×10^{-3}
1-15 ₅₀₀	(-HGT medium, no MTX)	18.0×10^{-3}
1-15 _{10,000}	10 μ M MTX	29.3×10^{-3}
1-15 _{10,000}	10 μ M MTX	49.0×10^{-3}
18B-9	50 nM MTX	14.3×10^{-3}
18B-9	50 nM MTX	14.4×10^{-3}
18B-9	(-HGT medium, no MTX)	14.3×10^{-3}
18B-9	(-HGT medium, no MTX)	14.4×10^{-3}
1	(-HGT medium, no MTX)	1.0×10^{-3}
1	(-HGT medium, no MTX)	0.7×10^{-3}

*tPA in the culture medium was assayed quantitatively in a radioimmunoassay as follows: Purified tPA and purified iodinated tracer tPA derived from melanoma cells were diluted serially to include concentration of 12.5 to 400 ng/ml in a buffer containing phosphate buffered saline, pH 7.3, 0.5 percent bovine serum albumin, 0.01 percent Tween 80, and 0.02 percent NaN₃. Appropriate dilutions of medium samples to be assayed were added to the radioactively labelled tracer proteins. The antigens were allowed to incubate overnight at room temperature in the presence of 1:10,000 dilution of the IgG fraction of a rabbit anti-tPA antiserum. Antibody-antigen complex was precipitated by absorption to goat anti-rabbit IgG Immunobeads (BioRad) for two hours at room temperature. The beads were cleared by the addition of saline diluent followed by centrifugation for ten minutes at $2000 \times g$ at 4° Celsius. Supernatants were discarded and the radioactivity in the precipitates was monitored. Concentrations were assigned by comparison with the reference standard.

The cell lines are as follows: Cell line "1" is an unamplified clone from the original set of four. "1–15₅₀₀" is an amplified subclone of cell line "1" which was amplified initially in 50 nM MTX to give 1–15 and then transferred for further amplification into 500 nM MTX. 1–15_{10,000} is subclone of 1–15₅₀₀ which has been further amplified in the presence of 10,000 nM MTX. Cell line 18B–9 is a subclone

11

of one of the original four detected which had been amplified on 50 nM MTX.

All of the amplified cells show increased levels of TPA production over that exhibited by the unamplified cell culture. Even the unamplified culture produces amounts of tPA greater than 0.5 pg/cell/day; amplification results in levels approaching 50 pg/cell/day.

What is claimed is:

1. A cell culture comprising methotrexate (MTX) sensitive recombinant host cells transformed with an expression vector comprising a first DNA sequence encoding a dihydrofolate reductase (DHFR) protein with a low binding affinity for MTX, and a second DNA sequence encoding

12

human tissue plasminogen activator (tPA), tPA encoded by said second DNA sequence, and an effective amplifying concentration of MTX.

2. The cell culture of claim 1 wherein said recombinant host cells are CHO cells.

3. The cell culture of claim 2 wherein said CHO cells are of the cell line CHO-K1(ATCC No. CCL 61).

4. The cell culture of claim 1 wherein the MTX concentration is between 20 and 500,000 nM.

5. The cell culture of claim 4 wherein said concentration is at least 100 nM.

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